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13. ABSTRACT (Maximum 200 Words)

Previous studies from our laboratory have shown that breast cancer patients can be successfully immunized with HER-2/neu (HER2) peptide vaccines resulting in epitope spreading and improved survival. In autoimmunity, epitope spreading is associated with tissue destruction and may explain why our patients had improved survival. Identifying those antigens to which epitope spreading occurred in this population of breast cancer patients may prove useful in developing cancer vaccine strategies. Our hypothesis is that these antigens may be tumor rejection antigens. The specific aim of this proposal was to identify potential "tumor rejection" antigens using sera derived from breast cancer patients immunized with a HER2 peptide-based vaccine who demonstrated epitope spreading and prolonged survival after active immunization. To achieve this objective, we used the technique of serological analysis of recombinant cDNA expression libraries (SEREX) to screen for tumor-specific antigens. The work was divided into 3 tasks (1) construction of breast cancer cDNA libraries from 2 breast cancer cell lines, (2) to perform SEREX to identify potential tumor rejection antigens, and (3) to identify the antigens using DNA sequencing. The first aim was achieved by constructing cDNA libaries from 2 lines, SKBR3 and MCF7. We have screened these libraries with patient and normal serum and have recovered several putative candidate antigens. Three has been sequenced and are called Homo sapiens hypothetical protein MGC2574, Sjogren's Syndrome Autoantigen B, and Recombining binding protein suppressor of hairless. These immune response to these proteins are being studied to determine whether they may be ture tumor rejection antigens.

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Introduction

A major clinical problem in the treatment of breast cancer is the prevention of relapse. We hypothesize that relapse may be prevented with a vaccine targeting immunogenic proteins expressed in breast cancer. Over the last decade several investigators have shown that breast cancer is immunogenic and many breast cancer antigens have been identified. However, despite a number of immunogenic proteins being identified, investigators still do not know which proteins must be targeted to cause breast cancer destruction, i.e. which are "tumor rejection" antigens? Our goal is to identify tumor rejection antigens to be formulated into a breast cancer vaccine designed to prevent recurrence. We have successfully immunized breast cancer patients with HER-2/neu (HER2) peptide vaccines (1, 2). Ninety-two percent of patients developed T-cell immunity to HER2. Of note, the majority (>75%) of the patients also developed "epitope spreading" to breast cancer antigens not contained within the vaccines. Most importantly, epitope spreading was associated with prolonged survival in this study. The largest group of patients enrolled in the study was stage IV HER2-overexpressing breast cancer patients. These patients were followed a median of 4.6 years (55 months) after completing their last vaccination. Of the 19 Stage IV breast cancer patients enrolled 61% are still alive. Expected survival of these patients, even with the use of Herceptin, is approximately 25 months (3). These breast cancer patients, with improved survival and epitope spreading, represent a population from which therapeutic breast cancer antigens can be identified. Both pre- and post-immunization sera specimens are available on these patients which can be used to identify immunogenic proteins derived from breast cancer cDNA libraries. The goal of this proposal is to identify unique antigens immunogenic in breast cancer patients who had both improved survival and epitope spreading. The identification of antigens will be done with a technique called serological analysis of recombinant cDNA expression libraries (SEREX). Our intent was to focus on those proteins that became recognized by the immune system only after immunization with HER2 peptide vaccines. The purpose of this study is to identify those antigens specifically targeted by the immune response following immunization with a HER2 peptide vaccine during the time in which the patient demonstrated improved survival. It is surmised that the immune response against antigens involved in tumor rejection will be highest during this period and the chances of identifying the antigens will be greater than at any other time. The results could potentially lead to the development of vaccines with greater efficacy in preventing breast cancer.

Body

The statement of work for this grant consisted of 3 tasks. The progress made in each specific task is described with reference to the figures immediately following the discussion.

Task 1: To construct two breast cancer cell cDNA libraries.

The original task was to create cDNA libraries from 2 breast cancer cell lines, SKBR3 and MCF-7. However based on critiques of the study, we have slightly altered that to include an additional library produced from the MDA-MB231 breast cancer cell lines. SKBR-3 is a HER2/neu over-expressing and estrogen receptor negative cell line, MCF-7 is a

HER2/neu non-expressing and an estrogen receptor positive cell line, and MDA-MB-231 is a metastatic HER-2/neu-overexpressing, estrogen receptor positive cell line. Total RNA was isolated from all of the cell lines using Ambion's RNAqueous – 4PCR RNA isolation kit. A representative example of RNA isolated from SKBR3 is shown in Figure 1A. Isolation of the messenger RNA (mRNA) from total RNA was done using Stratagene's Poly(A) Quik mRNA isolation kit produced high quality mRNA for library construction. The production of the library required 5μg mRNA from each cell line. The acquisition of 5 μg of mRNA was problematic for all of the cell lines. While we had initially anticipated 1 month for isolation of enough mRNA to construct the libraries, an additional two to four weeks was required. When sufficient mRNA was isolated, lambda phage cDNA libraries were constructed using Stratagene's ZAP Express cDNA synthesis kit. The quality of the cDNA generated was assessed by gel electrophoresis and PCR. Shown in Figure 1B is a representative example of production of the cDNA. Lanes 2-3 show the cDNA first strand synthesis and Lanes 4-5 show the second strand synthesis. Lanes 8-10 show the different fractions collected from the purifying column. We used the material collected in the fraction represented in Lane 9 which showed the highest concentration of cDNA which is observed as a smear over a broad weight range. Stratagene's Gigapack III Gold Packaging Extract was used to package the cDNA into phagemids. Each primary library consisted of $>8x10^5$ clones. Libraries were amplified once in XL1-Blue MRF` cells. The amplified libraries contained approximately 10^9 plaque forming units(pfu)/ml. Both of the SKBR3 and MCF-7 libraries have been completed. The final MDA-MB-231 library is still being developed. Significant problems developed during the production of the MCF-7 library and an additional 4 months were required to correct the problems. The main obstacle was the packaging was ineffective. Although we do not know the precise problems, we suspect that the reagents that we received from Stratagene were defective. Nonetheless, we completed the library and are in the process of beginning our screening. As a last step to verify that representative libraries have been produced, several plaque forming units derived from the libraries to confirm heterogeneity of the inserts. As shown in Figure 2, there was considerable heterogeneity amongst the insert sizes.

Task 2: To Perform SEREX screening to identify potential tumor rejection antigens.

Breast cancer patient serum from one of our previous clinical trials was used to screen the generated cDNA libraries. To reduce background during the SEREX assay, the patient serum was first cleared of E. *coli* antibodies using CnBr activated Sepahrose beads. The pre-cleared serum was used as the primary antibody source in the SEREX assay. Preclearing minimized the numbers of antibodies in the serum samples that were reactive against E. coli proteins.

To prepare nitrocellulose plaque lifts (i.e. membranes), XL1-Blue MRF` cells were incubated with the amplified SKBR3 library and then plated on NZY agar plates. Following a 7-8h hour incubation, the agar plates were overlaid with IPTG impregnated membranes, and further incubated overnight. These membranes were then screened using the breast cancer patient serum (1:200) as the primary antibody and goat α-human IgG (1:2500) as the secondary antibody. Following staining with BCIP/NBT, positive plaque-forming units (pfu) were further purified and isolated by picking and dilution plating.

Once certain that we had the positive clone, the clone was excised using XLOLR cells. PCR was done to check for homogeneity among like clones. A representative example of a positive clone is shown in Figure 3A. The clone (examples are circled) was derived from a single positive signal (not shown). Four of the clones were tested by PCR analysis as shown in Figure 3B. The four clones were all identical in size indicating that the enrichment for this particular clone was successful. A summary of the progress made with the screening for the libraries to date is shown in Table 1. The problems encountered, described above, have put us approximately 2-3 months behind schedule. Therefore, much of the screening has yet to be accomplished. Nonetheless, a significant level of screening of the SKBR3 library has been done and several potential proteins are currently being purified and sequenced.

Task 3: To identify potential tumor rejection antigens by sequencing the cDNAs recognized by post-immune serum.

Thus far we have several positive clones isolated and from those have identified 3 potential antigens. The first is a protein called Homo sapiens hypothetical protein MGC2574 (Accession # NM_024098). This protein is hypothetical and only known to exist as mRNA. An analysis of its predicted protein sequence shows significant homology to some voltage-gated potassium channels (4) and the TXK tyrosine kinase(5). It is expressed in both human and mouse. However, we found that this clone is reactive to both post-vaccination serum and pre-vaccination serum. We have continued to analyze this protein although because the intensity of staining in the pre-immune serum is less indicating that vaccination resulted in an elevation of the immune response to this protein. This protein has not been described in the SEREX databases and is therefore a novel finding. The second protein identified as an antigen in post-immune serum is Sjogren's Syndrome Autoantigen B (Accession # NM_003142). This is a target commonly observed in both Sjogren's Syndrome and Lupus (6). This is also a novel finding and has not been previously described. Investigations are ongoing to determine if immunity to this protein was induced as a result of immunization. The third protein identified, which has been previously described as an autoantigen is Recombining binding protein suppressor of hairless (RBPSUH) (Accession # NM_203284). There is evidence that this protein is a transcript factor (7). Studies are underway to determine if the immunity was induced by immunization. It is estimated that 800 plates will need to be screened during the primary screen in the SKBR3 library. Thus far 200 have been screened. An additional 175 plates have been screened during secondary screening and isolation/purification processes with the SKBR3 libraries. Screening will also begin soon on the MCF-7 and MDA-MB-231 cell lines.

Key Research Accomplishments

- Constructed 2 breast cancer cell lines for SEREX screening to fully accomplish Task 1.

- Completed approximately 30%-50% of the screening of the SKBR3 library and have identified 3 candidate antigens that we are further exploring as potential targets for future vaccine studies.

- One protein (Homo sapiens hypothetical protein MGC2574) has been identified as a potential antigen to which immunity was augmented following immunization. Two other proteins (Sjogren's Syndrome Autoantigen B and Recombining binding protein suppressor of hairless) have been identified and are being further analyzed.

Reportable outcomes

- Two breast cancer cell lines have been produced as a result of this project. This is significant because libraries, useful in SEREX, have not been available for these types of analysis.

- Based on our progress so far, an R21 (R21CA105270-01) was received to continue the research. We will continue to (1) develop the 3rd library, (2) screen the libraries, and (3) identify sequences from positive SEREX results. In addition, this grant will also provide for funding to test for pre-existing immunity in cancer patients.

- Two review articles are in press that both discuss our program of antigen discovery. Both of these review articles cite this grant a source of funding for our antigen discovery program. The citations of these articles are:

  - **Knutson KL** and Disis ML, 2004, Tumor antigen-specific T helper cells in Cancer Immunity and Immunotherapy. *Cancer Immunol Immunother* (In press).
  - **Knutson KL** and Disis ML, 2004, Molecular Identification of CD4 T Helper Epitopes. *Current Drug Targets-Immune Endo Metab Dis* (In press).

- Two novel proteins (Sjogren's Syndrome Autoantigen B and Homo sapiens hypothetical protein MGC2574) have been identified to which patients have generated antibodies. These will be entered into the SEREX database (http://www.eurice.de/eucip/serex.htm).

Conclusions

In summary, we have made significant progress toward achieving our overall objectives as outlined in the Statement of Work. Specifically we have completed production of the two breast cancer libraries and have made substantial progress in screening one of the libraries to come up with several candidate proteins. Three of the proteins have been identified and immunity was detectable in post-immune serum. There is some evidence to suggest that vaccination elevated immunity to one novel antigen. Currently, we have 2 other proteins we are in the process of identifying and determining if immunity was generated as a result of immunization. Due to some technical difficulties, we are somewhat behind on screening the MCF-7 libraries but these problems have been resolved and screening will initiate shortly. We have also generated a third library from another breast tumor cell, MBA-MD-231. Importantly, we have secured funding to continue the project and we are

still on track with our ultimate goal of identifying novel antigens to which immunity was generated in breast cancer patients, with improved survival, as a result of immunization. Although many tumor antigens have been identified in recent years, it is unclear as to which antigens are involved in tumor rejection. Our study is unique in that it allows us to potentially identify some of these antigens in patients who had apparent improved survival following immunization with a cancer vaccine. The development of multiantigen vaccine targeting biologically relevant antigens (i.e. tumor rejection antigens) may eventually allow the use of vaccines as an effective tool at preventing relapse in high risk breast cancer patients.

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	SKBR3	MCF-7	MDA-MB-231
Total Screens to date	400	0	0
# of identifications	3	0	0
# of hits	9	0	0
% screened	30%-50%	0	0

TABLE 1

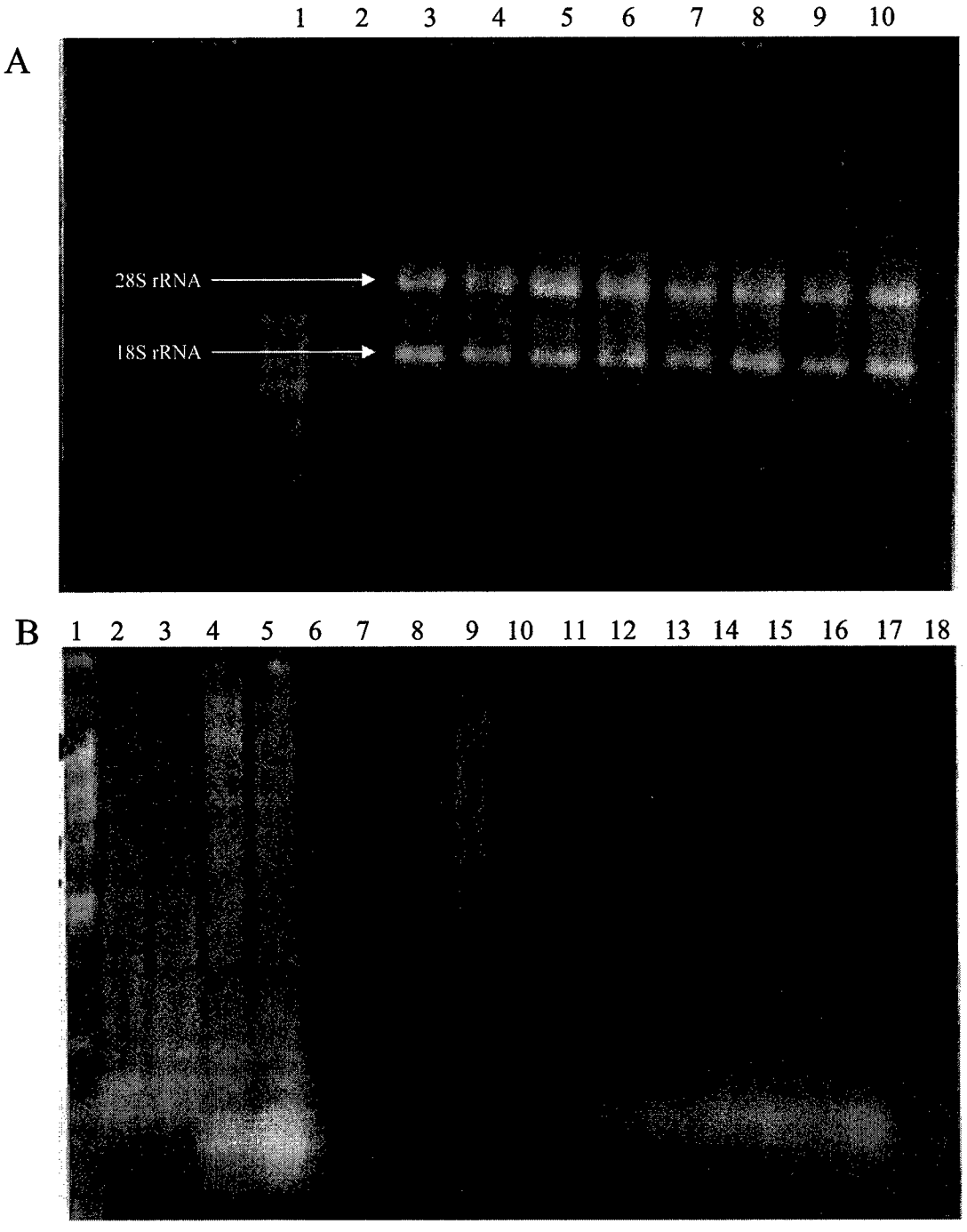

Figure 1: Production of cDNA from the breast cancer cell line SKBR3. Panel A shows 8 different preparations of total cellular RNA from SKBR3 demonstrating the integrity of the RNA. Panel B shows creation of the cDNA from SKBR3. Lanes 1-2 and 3-4 are the first and second strand synthesis reactions respectively. Lanes 8-17 show fractions collected during separation of the free nucleotide from the cDNA molecules. The cDNA library was prepared using the fraction depicted in Lane 9. Note that cDNA appears as a smear over a broad range.

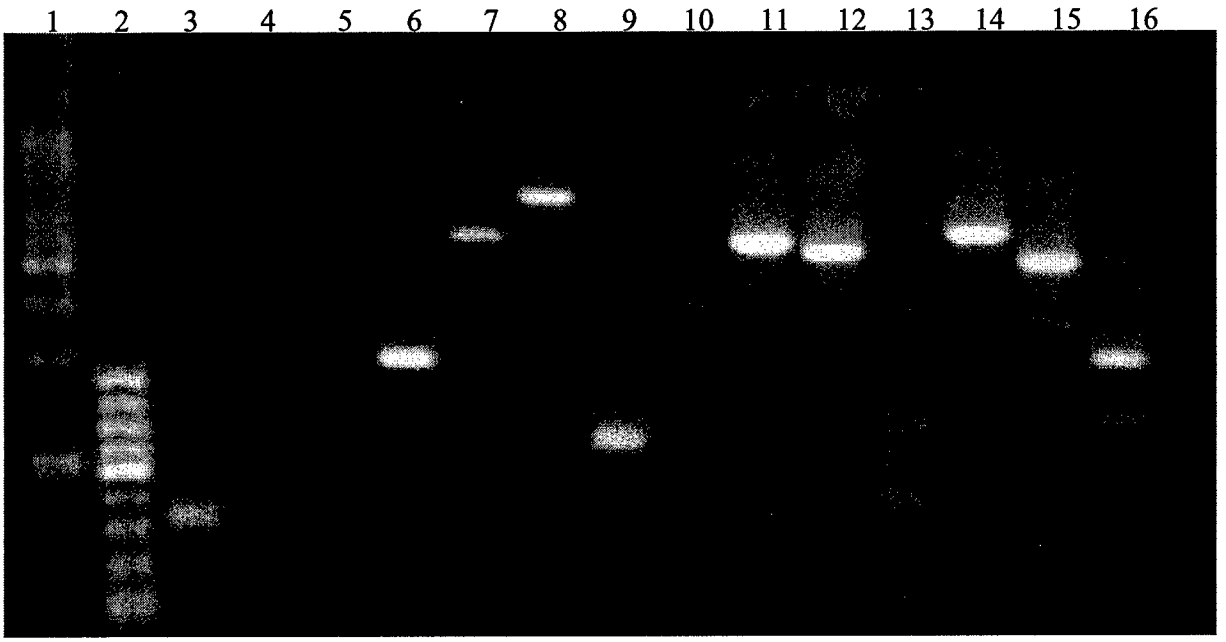

Figure 2: cDNA library created from the breast cancer cell line SKBR3. A sample of the SKBR3 library was subjected to PCR using primers, T3 and T7, specific for the phagemid and that flank the inserts. Lanes 1 and 2 are high and low range reference DNA. Lanes 3-16 show PCR analysis of plaques derived from plated bacteria infected with phagemid. Note that there is heterogeneity in the sizes of the inserts suggesting demonstrating that the library may be representative of the genes expressed in the cell line.

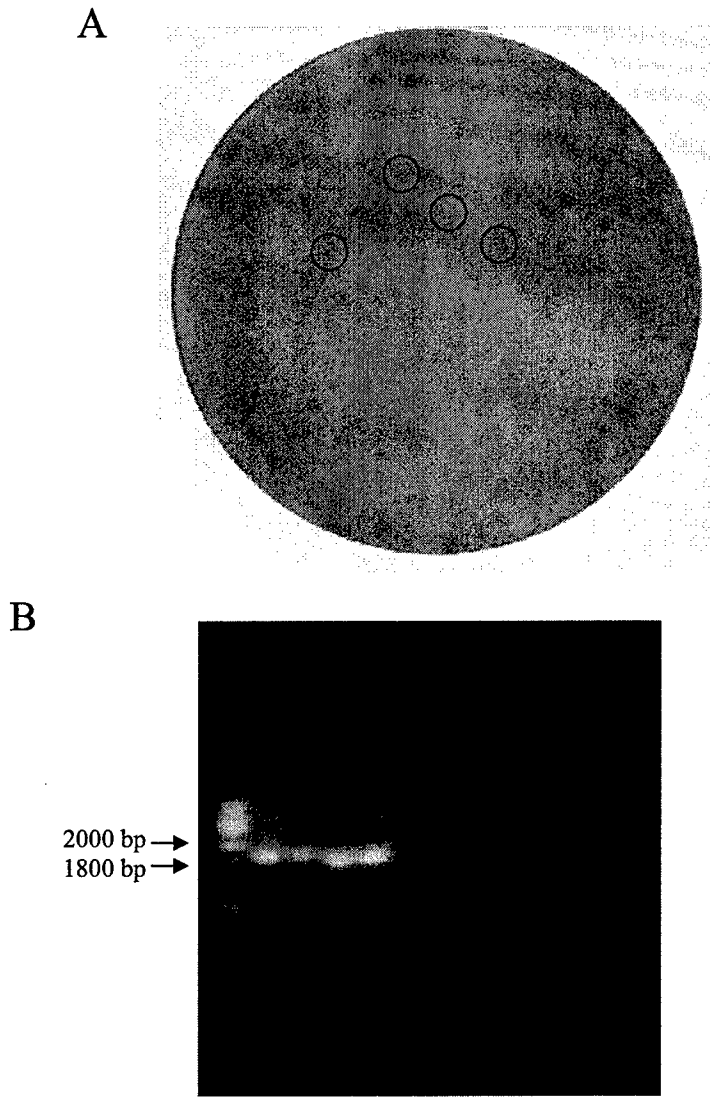

Figure 3. Phagemids expressing 1A1B-m can be enriched for purification. The phagemid expressing protein 1A1B-m was picked from its original plate and amplified in bacterial broths and subsequently replated to form plaques. As shown in Panel A, this process enriched the number of plaques that can be detected with the post-immune serum. Panel B shows the PCR analysis of 4 (circled in Panel A) of the plaques picked directly from the plate. These results suggest that the plaques containing the protein 1A1B-m can be enriched for identification.

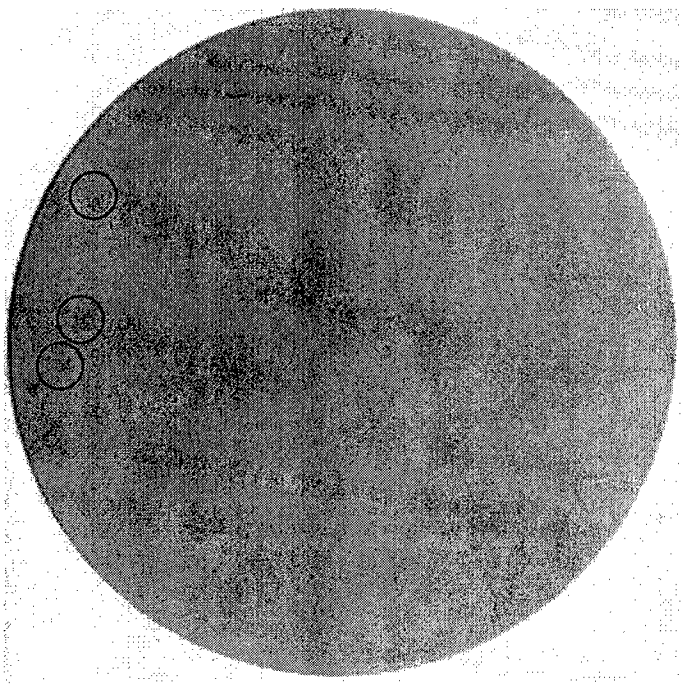

Figure 4. Phagemids expressing 1A1B-m are faintly detected in preimmune serum. The phagemid expressing protein 1A1B-m was picked from its original plate and amplified in bacterial broths and subsequently replated to form plaques. The plaques lifts were screened with pooled immune serum from pre-immune (i.e. prevaccine) serum. Note that the protein is also recognized by pre-immune serum indicating that immunity to it is not induced by vaccine. However, intensity of staining in much lower in preimmune serum indicating that prior vaccination leads to upregulation of immunity to this antigen. We will assess if vaccination resulted in the augmentation of this novel protein.